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STUDIES ON THE DENATURATION OF EGG ALBUMIN UNDER HIGH PRESSURE

By Keizo Suzuki*

A number of studies have been carried out on the denaturation of protein under high pressure. Bridgman[1] has reported that egg white coagulates completely at 7000 atm within 30 minutes, and Kiyama *et al.*[2] have confirmed that the coagulation occurs above 3880 kg/cm^2. Grant *et al.*[3] and Suzuki *et al.*[4] have found that SH groups are detected in the compressed sample of egg albumin. On the other hand, Johnson *et al.*[5] and Tongur[6] have reported that the thermal denaturation of proteins is retarded in a few examples by the pressure of about 1000atm.

In order to discuss the nature of denaturation of proteins in relation to pressure, especially to discuss the relation between these two opposing roles of pressure, *i. e.* acceleration and retardation, more abundant and more quantitative informations should be required.

In the present research, egg albumin was used, as a typical globular protein, and its rate of denaturation under different pressures and temperatures was studied from the measurement of concentration change due to precipitation induced by denaturation.

Experimentals

Preparation of egg albumin Egg albumin was prepared from hen's egg white by the method of Sørensen and Hørup[7], recrystallized three times, dialyzed against water untill free from ammonium sulfate, and stored as the stock solution in a refrigerator.

This stock was diluted with water and acetate buffer of pH 4.8 (except for the studies of the effect of pH) to a given protein concentration and a buffer concentration (usually 0.1 M), and used as a test solution.

It was found that the rate of denaturation is very sensitive to the freshness of egg albumin, even if stored in a refrigerator, so that newly prepared egg albumin was used within a week.

High pressure apparatus and procedures The schematic layout of the compressing apparatus is shown in Fig. 1. The experiments were carried out in the part A of the figure,

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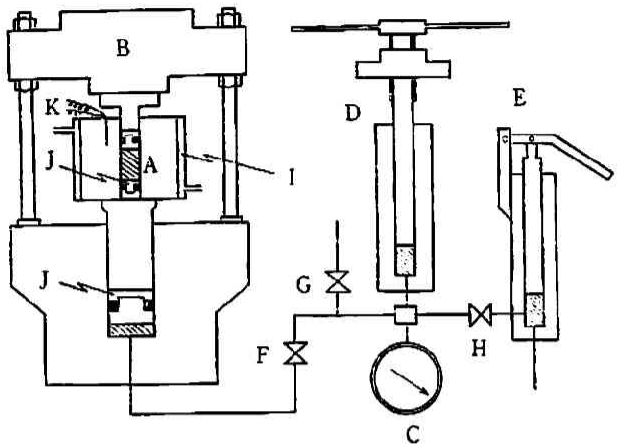

Fig. 1 Shematic layout of apparatus

A : high pressure chamber
B : compression apparatus
C : Bourdon type pressure gauge
D : injection pump
E : hand pump
F, G and H : high pressure valves
I : water jacket
J : self-tightening packing (Bridgman type)
K : thermister

where a test solution sealed into a polyvinyl chloride sack was charged. The reaction temperature was kept constant by circulating water from the thermostat through the jacket surrounding the vessel, but owing to the large heat capacity and heat conductance of the apparatus the temperature in the vessel was often found to differ from that of the thermostat. So the temperature of the vessel was measured directly by means of the thermister inserted in a small side-chamber of the vessel K.

After a definite time under compression, the sack containing the test solution was taken out and the sample was filtered, and then the protein concentration of the filtrate was determined by the colorimetric measurement of biuret reaction[8], corrected by the micro-Kjeldahl analysis of nitrogen[9], by means of an electron-tube-photometer.

Results

Effect of pH The effect of pH on the pressure denaturation of egg albumin was studied in the pH range 4.0～7.0. The effect was examined by applying about 5000kg/cm² for 10 minutes. In this experiment only, Merck's impulpable powder of egg albumin was used. The test sample contained *ca.* 0.7mg/ml of protein nitrogen, and its pH was adjusted with HCl or NaOH without using a buffer solution. After the application of pressure, the pH of the solution was brought to 4.8 with acetate buffer. After centrifugation, the nitrogen concentration in the supernatant was determined by the micro-Kjeldahl analysis.

The results are shown in Fig. 2. The nitrogen concentration in the supernatant takes a minimum value at about pH 4.8, the isoelectric point of egg albumin, namely, the rate of denaturation by pressure apparently reaches maximum here. Therefore, to minimize the complication due to the effect of pH, all other runs were carried out at pH 4.8.

Effect of pressing duration A number of experiments were carried out on the effect of

8) S. Nagaoka, T. Kaminaga and S. Araya, *J. Biochem.*, **41**, 37 (1954)
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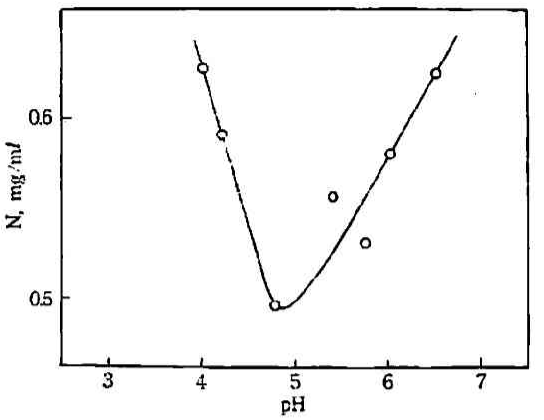

Fig. 2 Relation between nitrogen concentration in supernatant and pH

at 20°C, 5000 kg/cm² duration, 10 minutes and initial concentration of protein N, *ca.* 0.7 mg/ml

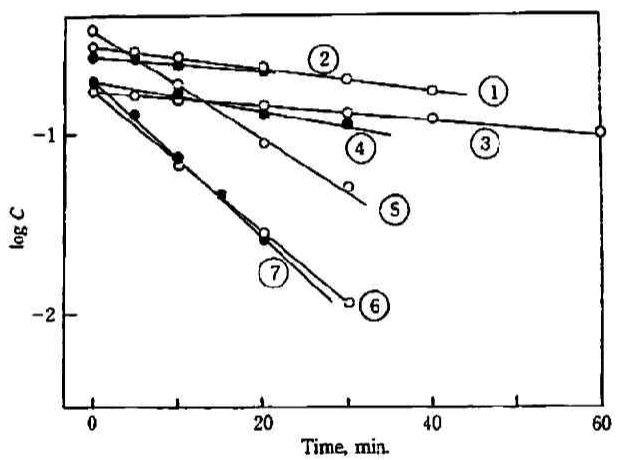

Fig. 3 Relations between logarithm of protein concentration C in supernatant and pressing duration

at pH 4.8

	Temperature, °C	Pressure, kg/cm²
①	65	4000
②	20	4500
③	65	3000
④	10	4500
⑤	15	5000
⑥	70	800
⑦	10	5000

It was found that the optical density of biuret reaction is linear to the protein concentraions, so that the values of optical density are taken directly as the measure of protein concentration in this figure.

pressing duration on the denaturation of egg albumin. A representative part given in Fig. 3 shows that the plots of the logarithm of the protein concentration in the supernatant against pressing duration constitute straight lines for the given pressures and temperatures, hence the denaturation of egg albumin by heat or pressure is of the first order.

Reversibility of denaturation Although it is found in a few examples that their denaturation by heat or pressure is reversible, yet under the present experimental conditions such a case is not found. No difference was found between the sample filtered immediately after the application of pressure and that filtered after being stored 2 or more days in the refrigerator at the isoelectric point or at more alkaline side of pH.

Effect of pressure The effect of pressure at constant pressing time was investigated at several temperatures. A given pressure was applied for 5 or 10 minutes to an egg albumin solution with M/10 acetate buffer of pH 4.8, containing *ca.* 0.5～1.5 mg of protein nitrogen per ml, and the remaining protein in the supernatant was determined. The results below 50°C as given in Fig. 4 show similar patterns; above a certain pressure (about 4000 kg/cm²), further increase in pressure results in a rapid increase of the precipitation up to a second point which varies with

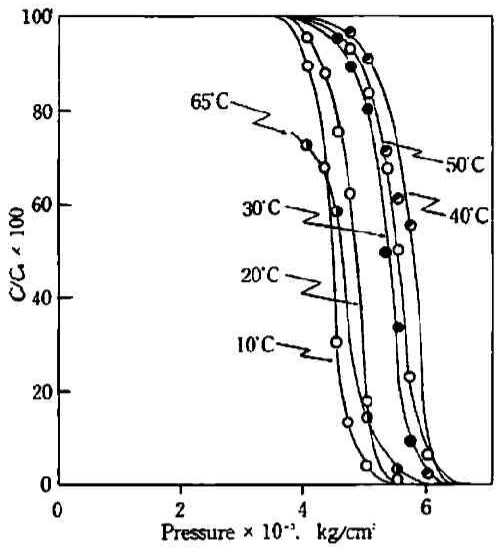

Fig. 4 Relations between the proportion of the protein concentration in supernatant C to the initial concentration C_0 and pressure

at pH 4.8, duration 10 min.,
initial concentration of N, *ca.* 1.1 mg/ml

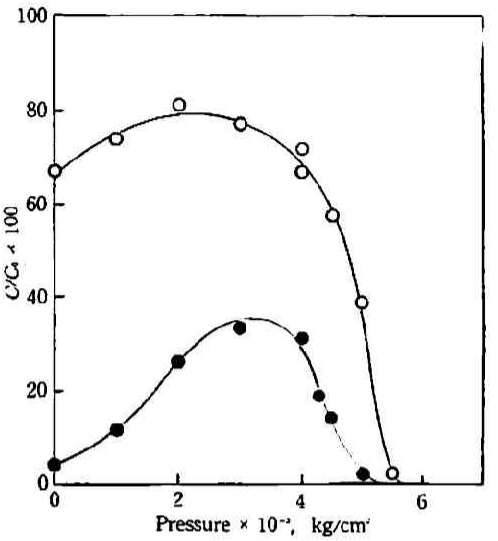

Fig. 5 Relations between the proportion of the protein concentration in supernatant C to the initial concentration C_0 and pressure

at pH 4.8, duration 20 min.,
initial concentration of N, *ca.* 1.1 mg/ml

Temperature, °C
○ : 65
● : 70

the temperature, above which the protein coagulates completely and no protein remains in the supernatant.

The effect of pressure above 50°C is much complicated, due to the thermal denaturation, which will be discussed later.

Effect of temperature As shown in Fig. 4, the increase in temperature shifts the concentration-pressure curve to the right side in the range 10～40°C, namely it decreases the rate of denaturation. But the relation is reciprocal above this temperature. Another effect is that the increase in temperature lessens the slope of the curve slightly in the whole temperature range of the experiment

Effect of temperature and pressure at relatively high temperature Approximately above 60°C, the so-called thermal denaturation occurs at atmospheric pressure. Fig. 5 shows the effects of pressure at 65 and 70°C at a definite pressing duration. In this experiment, considerable amounts of protein coagulates before the pressure reaching to an appointed value, and after releasing pressure. These values are determined in a blank test and are substracted from the experimental values. As shown in the figure, the increase in pressure rather increases the protein concentration in the supernatant below about 3000 kg/cm²; in other words, denaturation was retarded by pressure. But the curves corresponding to the range above 4000 kg/cm² show the similar paterns to the cases at lower temperatures; pressure causes a rapid decrease in protein concentration in the supernatant to zero, above a certain pressure.

Considerations

From the experimental results mentioned above, it seems that there are two features of denaturation processes under high pressure below and above 40°C respectively. As shown in Fig. 6, the plot of the logarithm of the rate constant against the reciprocal of the absolute temperature is found to be consisted of two straight lines for the pressure around 4500 kg/cm², which coincide together at the point corresponding to about 40°C.

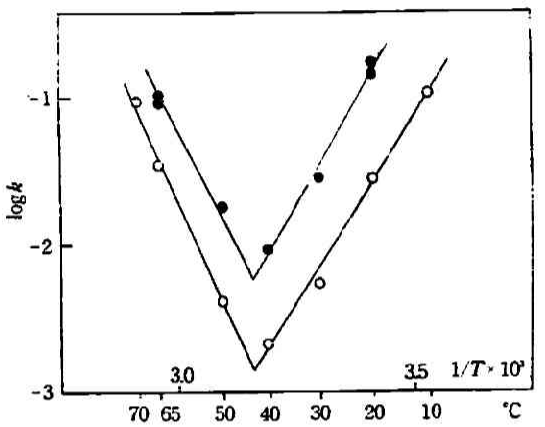

Fig. 6 Relations between logarithm of rate constant k (min^{-1}) and reciprocal of absolute temperature T, at pH 4.8

Pressure, kg/cm²
○ : 4500
● : 5000

The values of the activation enegy E are calculated by using the Arrhenius relation, from the slopes of each straight line which was obtained from two series of experiments carried out under 4500 and 5000 kg/cm² respectively. The values of E calculated and listed in Table 1 show that under these high pressure conditions, the activation energy E takes positive value in the range above 40°C and negative value in the range below 40°C.

Table 1 Apparent activation energy, E

Pressure kg/cm²	Activation energy, kcal/mole	
	10~40°C	40~70°C
4500	−24	+33
5000	−25	+31

The relation between the rate constant k and pressure P is represented by the equation:

$$\frac{\mathrm{d}\ln k}{\mathrm{d}P} = -\frac{\Delta V^*}{RT},$$

where ΔV^* is the molal volume change on activation, P the pressure, R the gas constant and T the absolute temperature. So the values of ΔV^* can be calculated from the slope of each line obtained by plotting the logarithm of rate constant against the pressure, as shown in Fig. 7, and are listed in Table 2.

Table 2 Molal volume change on activation, ΔV^*

Temperature, °C	10	20	30	40	50	65	70
ΔV^*, cc/mole	−92	−83	−81	−79	−77	−55	−38

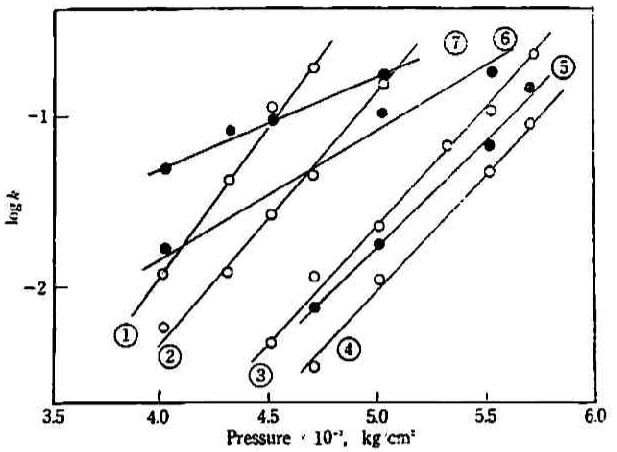

Fig. 7 Relations between logarithm of rate constant k (min^{-1}) and pressure at pH 4.8

	Temperature, °C
①	10
②	20
③	30
④	40
⑤	50
⑥	65
⑦	70

In these high pressure regions above 4000 kg/cm², the values of ΔV^* take negative values in all cases, but their absolute values tend to decrease with the increase in temperature. Especially in the case where the thermal denaturation under atmospheric pressure is remarkable, the values of ΔV^* decrease much more.

In Fig. 8 the logarithm of rate constants are plotted against pressure in a wide range of

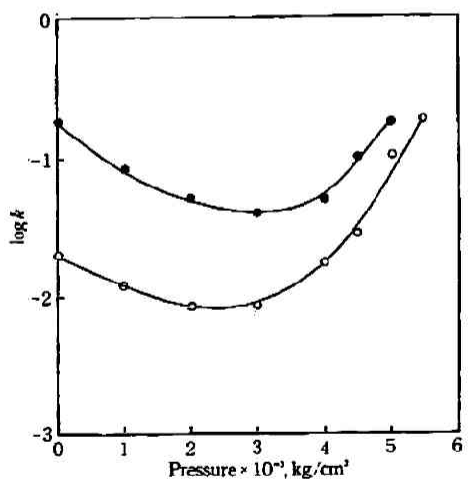

Fig. 8 Relations between logarithm of rate constant k (min^{-1}) and pressure at pH 4.8

	Temperature, °C
○ :	65
● :	70

pressure (0～1000 kg/cm²) at 65 and 70°C where evidently the thermal denaturation occurs (*cf.* Fig. 5). Probably as the effect of two types of denaturation due to heat and to pressure overlap, the linear relation does not hold. But for convenience, the activation energy E at a certain pressure and molal volume on activation ΔV^* in a certain range of pressure are calculated from Fig. 8, the values being shown in Table 3. It is noticeable that the values of E are positive and decrease with the increase in pressure, while the values of ΔV^*, changing their sign below and above 3000～4000 kg/cm², decrease with the increase in pressure.

The kinetic results obtained above from the experiments and summarized in Fig. 9 indicate

Table 3 Apparent activation energy, E and apparent molal volume change on activation ΔV^* at higher temperature

Pressure, kg/cm²	E, kcal/mole	ΔV^*, cc/mole	
		at 65°C	at 70°C
0	102		
800	87	+14	+24
2000	83		
3000	69	−55	−38
4500	33		

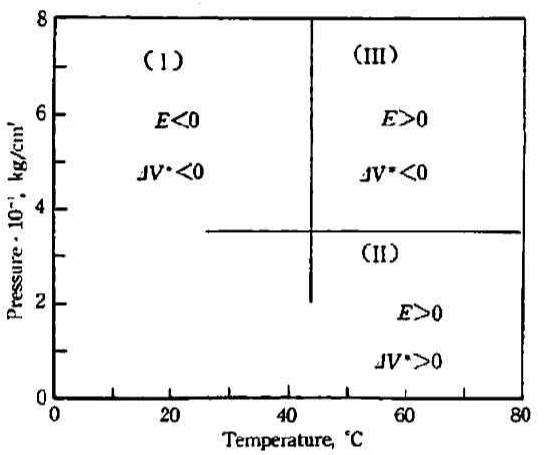

Fig. 9 Variation of activation energy and volume in activation in relation to temperature and pressure

apparently three kinds of denaturation, namely, (I) that at relatively high pressure and low temperature where $E<0$ and $\Delta V^*<0$, (II) that at relatively low pressure and high temperature where $E>0$ and $\Delta V^*>0$, and (III) that at high pressure and high temperature where $E>0$ and $\Delta V^*<0$.

Whether these differences mean the different denaturation paths or whether these are ascribed to a complicated activation mechanism of protein denaturation, is the most fundamental problem and the author has some conceptions about this. But this classification is based merely on the observation of the precipitation due to denaturation, and now the author, carrying out some experiments, where SH groups, viscosities and electrophoretic behaviors of native and denatured egg albumin are measured, will make full discussion later, taking all those results into consideration.

The author has a great pleasure in expressing his sincere thanks to the late Prof. Ryo Kiyama for his valuable guidance and encouragement in the beginning of this work.

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